Wang et al., Supplemental Material

Supplemental Methods:

Generating RFS-*myc*^{WT}, RFS-*myc*^{T58A} and RFS-*myc*^{S62A} mice: murine c-*myc*^{WT}-*HA* or phosphorylation mutant c-*myc*^{T58A}-*HA* or c-*myc*^{S62A}-*HA* cDNAs (Arnold and Sears, 2006) were inserted into the previously described ROSA26 vector pBigT. This cassette was subcloned into the pROSA26PA plasmid for homologous recombination into the *ROSA26* genomic locus in 129 ES cells. Correct gene targeting in selected ES clones was identified by PCR with primers from *ROSA26* flanking (F-SL347) and Splice Acceptor (R-SL348) (see Supplementary Table 1). Positive ES cell clones were verified by sequencing through the 5' *loxP* site into the middle of the knocked-in c-*myc* cDNA. Correctly targeted and sequence-confirmed ES clones were injected into C57BL/6 blastocysts to obtain chimeric mice. Germline transmission was obtained by crossing with C57BL/6 mice to establish homozygous knock-in RFS-*myc*^{WT}, RFS-*myc*^{T58A} and RFS-*myc*^{S62A} strains.

Genotyping: PCR analysis for genotype uses primers pROSA884F (Supplementary Fig. 1B, primer 1) and pBigT86R (Supplementary Fig.1B, primer 3) (see Supplementary Table 1 for primer sequences). PCR amplification results in a 300-bp DNA fragment. The wild type (untargeted) mice were identified by primer 1 and primer pROSA1447R (Supplementary Fig. 1B, primer 2). PCR amplification results in a 563 bp DNA fragment. Wap-*Cre* was detected with primers WAP-1 and WAP-2. The PCR temperature profile was 95^{o}C for 30 seconds, 50^{o}C for 30 seconds, and 72^{o}C for 30 seconds with extension of the last cycle for 10 minutes at 72^{o}C. Cre-mediated recombination in RFS-*myc*/Wap-*Cre* mice was detected with primer 1 and Myc224R (Supplementary Fig. 1B, primer 5) yielding a 1.2kb PCR product, and the un-recombined allele was detected with primer 1 and pBigT254 (Supplementary Fig. 1B, primer 4) (product size 470 bp).

Cell Line: 293 cells were acquired from the ATCC in 2002. They have not been tested for authentication since purchase.

RNA analysis: RNA was isolated from mammary gland using TRIzol reagent (Invitrogen) according to manufacturer's protocol. RNA was purified using RNeasy mini kit (Qiagen). cDNAs were made from DNaseI-treated RNA using High Capacity Reverse Transcription (RT) kit (Applied Biosystems) or M-MLV RT Kit (Invitrogen) according to manufacturer's protocol with random primers. Quantitative RT-PCR analysis was done on a StepOne qRT-PCR machine (Applied Biosystems) according to manufacturer's preset PCR cycle conditions. Primers used to detect total c-*myc* were MycFM1 and MycRM1 (see Suppl. Table 1). Ectopic c-*myc* was detected with MycFM1 and HA-R.

Antibodies: HA-11 (G036, Applied Biological Material) 1:1000; Bim/BOD (Stressgen Bioreagents, BC Canada) 1:1000; c-Myc Y69 (ab32072, Abcam) 1:1000; c-Myc Phospho-T58 (Y011034, Applied Biological Material) 1:500; c-Myc Phospho-S62 (E71-161, BioAcademia Inc) 1:200; ß-actin (A5441, Sigma) 1:10,000; pericentrin (PRB-432C, Covance) 1:1000; E-Cadherin (Cell Signaling, #4065)1:100; Ki67 (Novocastra, NCL-Ki67-MM1)1:200; Mouse Keratin 14 (Covance, PRB-155p) 1:1000; Cytokeratin 8/18 (Fitzgerald, 20R-CP004) 1:100; alpha Smooth Muscle Actin (SMA) (abcam, ab5694) 1:400.

Western analysis: Mouse mammary gland samples were lysed by homogenizing in EBC buffer (50mM Tris pH8.0, 150mM NaCl, 0.5% NP-40, 0.1% SDS) with protease and phosphatase inhibitors. Lysates were separated by SDS-PAGE and transferred to Immobilon-FL (Millipore, Beillerica, MA). AquaBlock (EastCoast Bio) was used to block membranes. Primary antibodies were diluted in AquaBlock-PBST buffer (50% AquaBlock, 50% PBS and 0.05% Tween-20) at indicated dilutions and detected by secondary anti-mouse or anti-rabbit near-infrared fluorescent dyes AlexaFluor 680 (Molecular Probes, Eugene, OR) or IRDye 800 (Rockland, Philadelphia, PA) with a dilution of 1:10,000 in AquaBlock-PBST buffer. Immunoblots were visualized via LI-COR Odyssey Infrared Imager (Lincoln, Nebraska) that can simultaneously detect Fluor 680 and IRDye 800 anti-rabbit and anti-mouse secondary antibodies. Quantification of western blots was done using LI-COR Odyssey Infrared software version 1.2, which is linear over 4 orders of magnitude.

Preparation of primary mouse embryo fibroblasts (MEFs) and mammary epithelial cells (MECs): For MEFs, 13.5d embryos were minced and incubated in Trypsin/EDTA for 15 minutes, spun at 1000RPM for 5 minutes, resuspended in DMEM 20% FCS. Cells were grown overnight, placed in fresh media and incubate for another 24 hours, at which time they were frozen. Thawed cells were considered passage 1 MEFs. For MECs, mammary glands were minced in MEBM/MEGM media (Clonetics) containing 1X collagenase/hyaluronidase enzyme solution. After 6 hours of digestion, released cells were cultured in MEBM/MEGM media. When MEF or epithelial cells reached about 80% confluent, they were used for immunofluorescence, half-life measurements, and centrosome detection and chromosome preparation. MEF and mammary epithelial cells were infected with Ad-Cre to induce Myc expression 48 hours prior to analysis.

Cycloheximide half-life: Primary MEFs or MECs (1-2 passages) were infected with Ad-Cre at MOI of 700 (MEF) or 200 (MEC) to induce Cre-dependent Myc expression for 48 hours and then starved in 0.1% FBS for another 48 hours. Cells were treated with 10 μg/ml of cycloheximide and equal numbers of cells were harvested at each time point. Cells were lysed in 1ml Ab lysis buffer (Malempati et al., 2006) per 100mm culture-dish. HA tagged WT, T58A or S62A Myc was immunoprecipitated with HA antibody and western blotted with Y69 Myc antibody. Quantification of western blots was done using LI-COR Odyssey Infrared software version 1.2. c-Myc half-life was calculated from exponential line equations.

Chromosome spreads: Cells were incubated at 37 °C for 2 hrs in MEBM/MEGM media containing 0.1μg/ml colcemid. Then cells were placed in 0.56% potassium chloride solution for 5 minutes and fixed in a freshly prepared 3:1 mixture of methanol and glacial acetic acid at 4 °C for 20 min. The cell suspension was then dropped across the surface of a slide, and cells were spread and allowed to dry on the surface. The slides were stained with Giemsa.

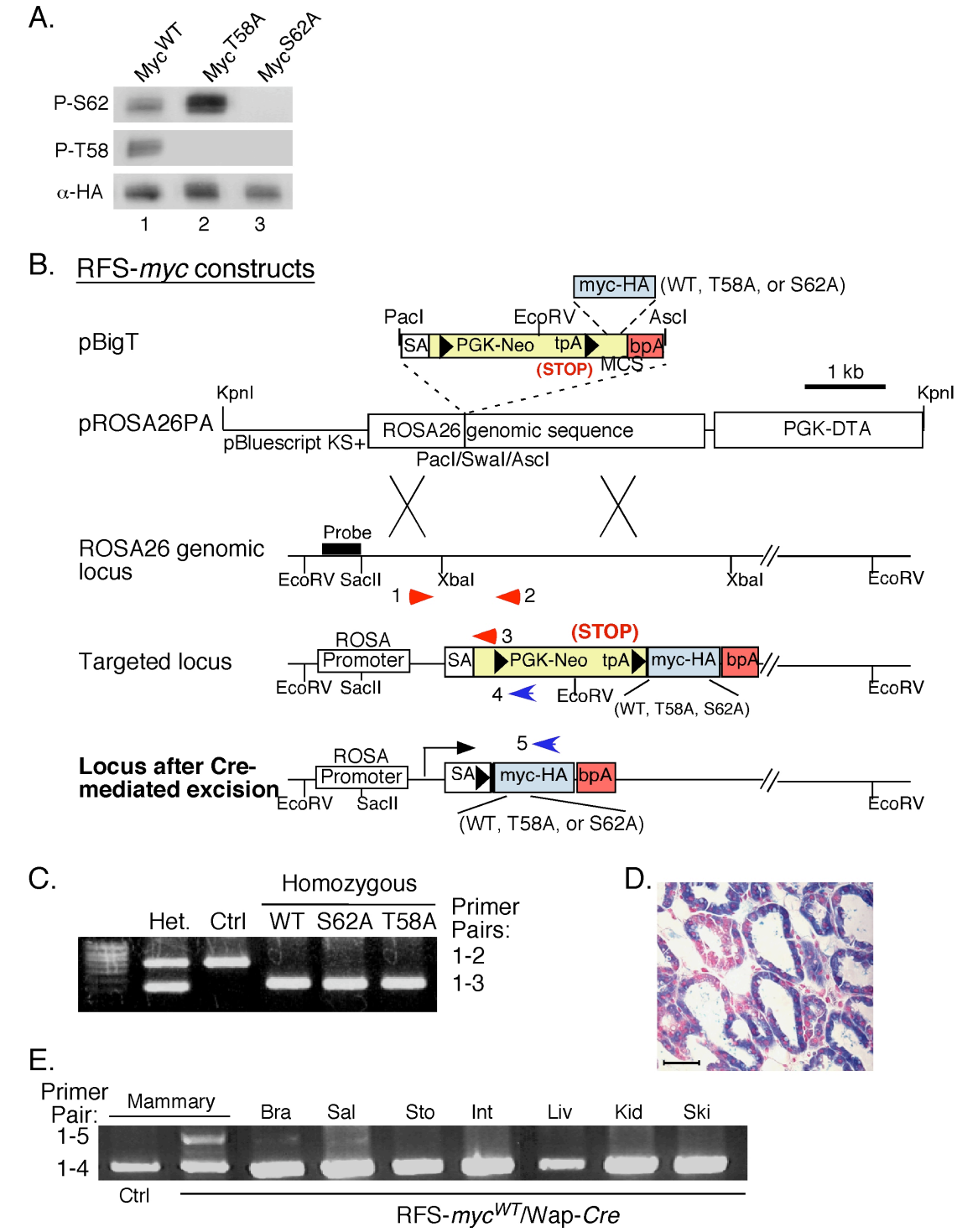
Centrosome staining: Cells were fixed in 2.5% paraformaldehyde, 25 mM $MgCl_2$, and PBS for 10 minutes at room temperature. The slides were then washed with 0.3 M

glycine/PBS, permeabilized in 0.2% Triton X-100/PBS, and incubated overnight with pericentrin antibody (Covance) diluted 1:1000 in 5% goat serum/PBS. The antibody complexes were detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Roche Applied Science) and DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). The number of centrosomes per mitotic cell was quantified.

Suppl. Table 1: Primers

Name	Sequence
F-SL347	5'-CCTAAAGAAGAGGCTGTGCTTTGG-3'
R-SL348	5'-CATCAAGGAAACCCTGGACTACTG-3'
pROSA884F	5'-AAAGTCGCTCTGAGTTGTTAT-3'
pBigT86R	5'-GCGAAGAGTTTGTCCTCAACC-3'
pROSA1447R	5'-GGAGCGGGAGAAATGGATATG-3'
WAP-1	5'-TAGAGCTGTGCCAGCCTCTTC-3'
WAP-2	5'-CATCACTCGTTGCATCGACC-3'
Myc224R	5'-GCAGCGAGTCCGAGGAAGG-3'
pBigT254	5'-GCCTTGGGAAAAGCGCCTC-3'
MycFM1	5'-CCACAGCAAACCTCCGCACAG-3'
MycRM1	5'-CCTTTTCAGAGGTGAGCTTG-3'
HA-R	5'-CTGGAACATCGTATGGGTACC-3'
GAPDH-F	5'-ACGGCCGCATCTTCTTGTGC-3'
GAPDH-R	5'-GTGCAGGATGCATTGCTGAC-3'
Aurora-B-F	5'-GCTTCGCCGAGAGATCGA-3'
Aurora-B-R	5'-AGAGTTGAAGGATGTTGGGATGTT-3'
Bub1b-F	5'-GGCCATGTTGTTTGGTACCAGTA-3'
Bub1b-R	5'-TTCGCTGTGTTGGAGAAGATTC-3'

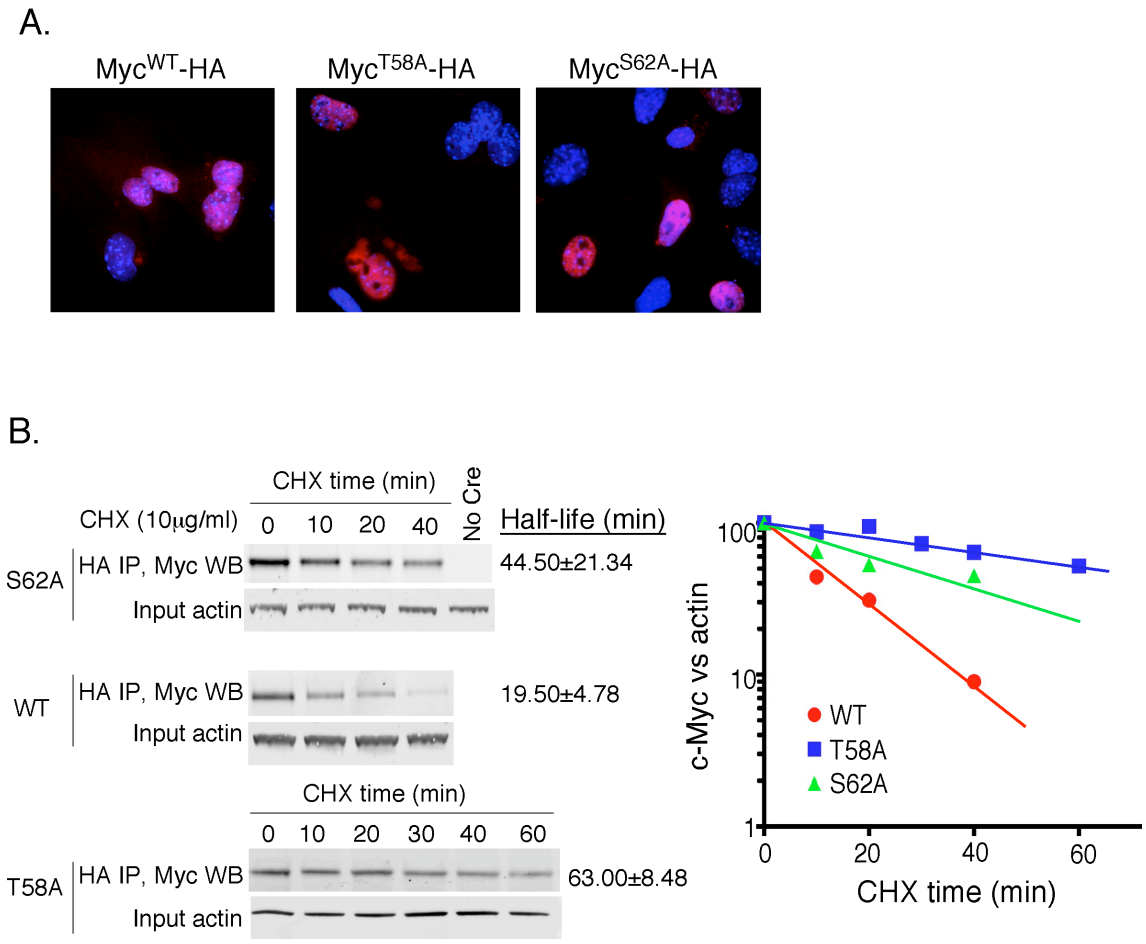
Wang Suppl. Fig. 1



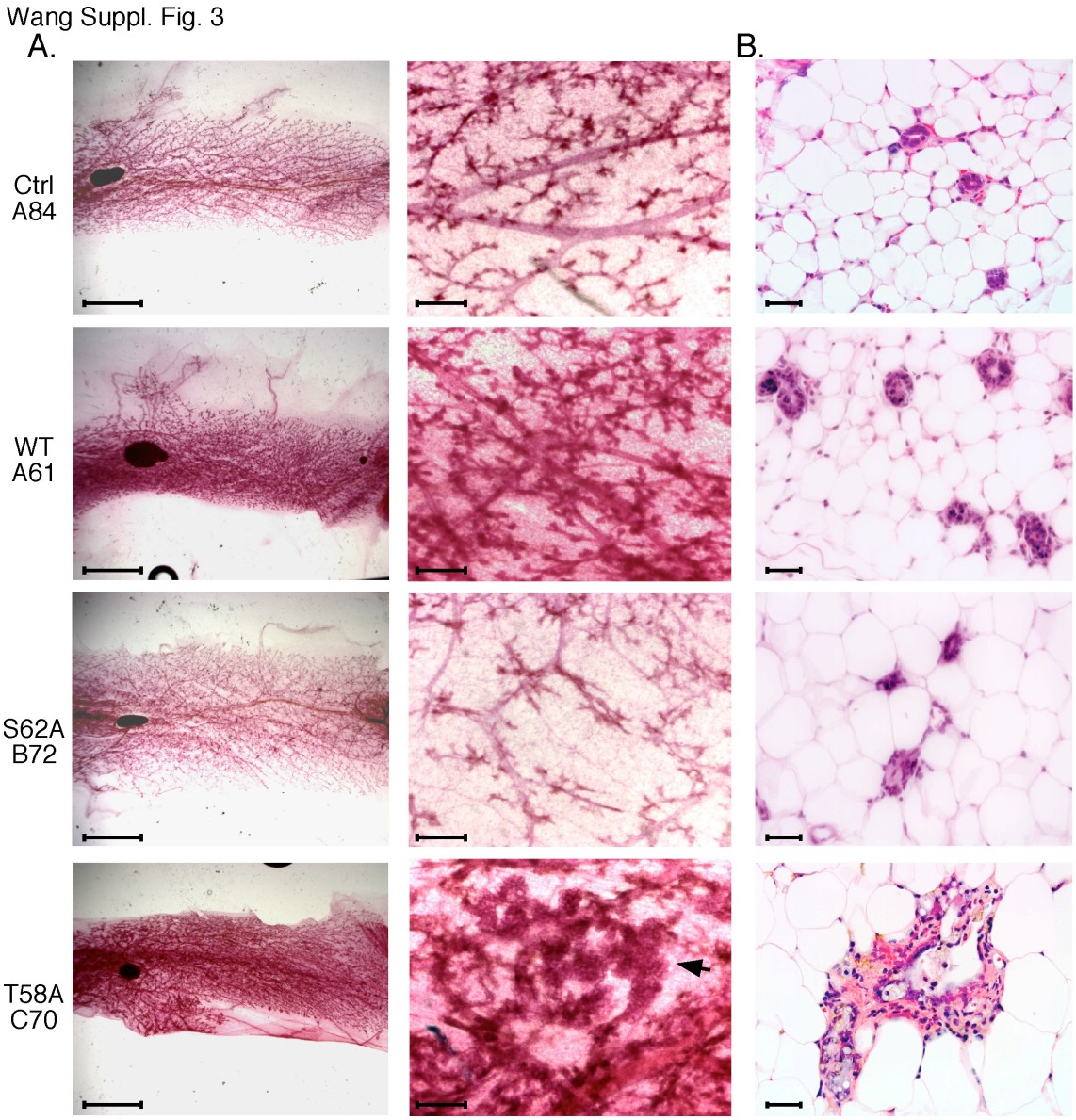
Suppl. Figure 1. Generation of Cre-conditional mice that express c-Myc^{WT}-HA, c-Myc^{T58A}-HA or c-Myc^{S62A}-HA from the *ROSA* locus. A. T58 and S62 phosphorylation status of c-Myc^{WT} and the c-Myc^{T58A} and c-Myc^{S62A} phosphorylation

mutants. Protein lysates from 293 cells stably expressing HA-tagged c-Myc^{WT}, c-Myc^{T58A} or c-Myc^{S62A} were analyzed by western blotting with either the HA antibody for total ectopic c-Myc or the phospho-S62 or phospho-T58 antibody as indicated. Blots were dual probed with phospho-Myc antibodies and the HA antibody. **B.** Strategy for generating conditional *c-myc* knock-in mice. cDNAs encoding murine *c-myc^{WT}-HA, c-myc^{T58A}-HA* or *c-myc^{S62A}-HA* were cloned into the multi-cloning site of pBigT. Black arrowheads indicate *LoxP* sites, *tpA* is a transcription stop sequence. This was subcloned into pROSA26PA. Targeted homologous recombination at the *ROSA26* locus is shown. Primer sets to detect correct targeting (1-3) and Cre-mediated genomic recombination (1, 4-5) are shown. **C.** Genotyping by PCR with primers shown in B using tail DNA from a heterozygous RFS-*myc^{WT}* mouse (Het.), control wild-type mouse (Ctrl), or homozygous RFS-*myc^{WT}*, RFS-*myc^{S62A}* or RFS-*myc^{T58A}* mice. **D**. X-gal staining showing Cre-mediated recombination efficiency in mammary epithelial cells. Homozygous RFS-*myc^{WT}*/WAP-*Cre* mice were crossed with the Cre-inducible ROSA26R lacZ reporter mice, R26R (Soriano, 1999). Mammary gland from a Wap-*Cre* positive progeny that had undergone one pregnancy and 12 days of lactation to activate the Wap promoter was stained with X-gal in whole mount and sectioned. LacZ expression stains blue. Scale bar is 50μM. **E**. Genomic DNA was extracted from the indicated tissues (Bra=Brain; Sal=Salivary gland; Sto=Stomach; Int=Intestine; Liv=Liver; Kid=Kidney; Ski=Skin) from homozygous RFS-*myc^{WT}*/WAP-*Cre* or control mice (ctrl) without WAP-*Cre* five months after the 3rd pregnancy. Cre-mediated recombination (upper band) was detected by PCR.

Wang Suppl. Fig. 2

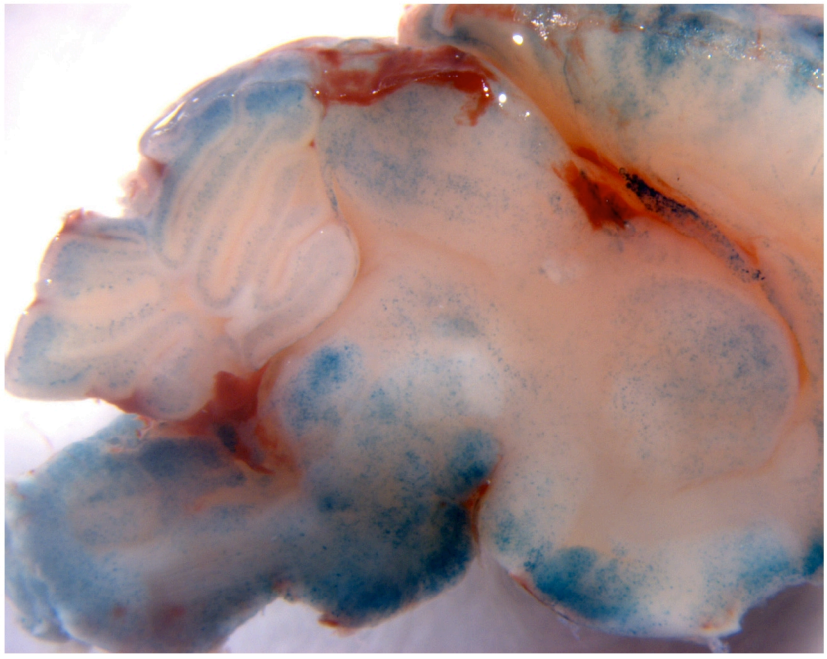


Suppl. Figure 2. Nuclear expression and half-life of c-Myc^{WT}, c-Myc^{T58A} and c-Myc^{S62A} in primary MEFs isolated from RFS-*myc*^{WT}, RFS-*myc*^{T58A} and RFS-*myc*^{S62A} mice. A. Immunofluorescence staining of ectopic c-Myc expression with HA antibody in primary MEFs prepared from RFS-*myc*^{WT}, RFS-*myc*^{T58A} or RFS-*myc*^{S62A} embryos (Red: anti-HA, Blue: DAPI). MEFs were infected with adenovirus (Ad)-Cre for 48 hours before staining. **B**. Ectopic c-Myc protein half-life was analyzed. Primary RFS-*myc*^{WT}, RFS-*myc*^{T58A} and RFS-*myc*^{S62A} MEFs infected with Ad-Cre were treated with cycloheximide. Cells were harvested and immunoprecipitated with HA antibody from equal cell counts at the indicated time points. Expression of c-Myc-HA and input Actin was analyzed by western blotting and quantified by LICOR scanner. Actin-normalized MycWT, T58A, or S62A expression across time points (with time zero set at 100) was graphed on semi-log plot and half-life was calculated by Excel exponential line function. Average half-life ± SD from three independent experiments is indicated.



Suppl. Figure 3. Altered mammary gland density with expression of c-Myc^{WT}, c-Myc^{T58A} and c-Myc^{S62A} at five months parous. The 4th glands from the indicated strains of mice five months parous after the 3rd pregnancy were analyzed by whole mount. Solid, darkly staining areas are lymph nodes. Arrow in bottom middle panel indicates hyperplastic foci. Scale bars are 5 mM (left column) and 200 μM (right column). Images shown are representative of 3 mice per genotype. **B**. As in A, but H&E stained sections. Scale bars are 50μM. Images are representative of 3-4 mice per genotype.

Wang Suppl. Fig. 4



Suppl. Figure 4. Spurious expression of Cre in the brain of Wap-*Cre* mice. ROSA26R lacZ reporter mouse was crossed with homozygous RFS-myc^{WT}/WAP-*Cre*. Wap-*Cre* positive progeny were analyzed at embryonic day E17, and post-natal days P1, P3 and P12. X-gal staining showing Cre-mediated recombination was first detected at P3 and is shown in a 12-day old mouse brain.